REMARKS

Status of the Claims

Claims 1-4 and 44 have now been canceled without prejudice or disclaimer. Applicants reserve the right to pursue these claims in subsequent and pending applications. Claims 7-30 were withdrawn by the Examiner in the Office Action. Claims 45-49 have now been newly added. Previously presented claims 5-6 and 31-43 as well as new claims 45-49 are now pending.

Amendment of Claims

Claims 5 as amended is an independent claim and claims 31, 33-36 and 38 were amended to depend from claim 5.

Claim 36 was amended in responsive to the Examiner's rejection of the claim as being indefinite.

Claim 42 as amended is also an independent claim.

Claims 31 and 33-34 were amended by the addition of the word "thereof." This amendment achieves consistency in the language of the claims (i.e. claims 5, 31-36, 38-40, 42, 45-49) that refer to "a monoclonal antibody or fragment thereof." This amendment does not change the scope of the claims.

Claims 32, 35, 37, 39-40 were amended by the substitution of "the" with "a" and/or the deletion of "a" as indicated to achieve consistency in the language of the claims. This amendment does not change the scope of the claims.

None of the amendments introduce new matter.

Claim Objections

Rejections under 35 U.S.C. § 112, second paragraph

Claims 4 and 36-37 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. We note that we assume that in section 8.B. of the Office Action the Examiner referred to claim 36 and not to claim 35 as stated. Applicant canceled claim 4, rendering its rejection moot. Applicant amended claim 36 in response to this rejection. The amendment adds a claim limitation that clarifies the role of an antibody of the invention in the claimed pharmaceutical composition for gene therapy treatment. The role of the antibody is not to replace an absent or defective gene, but to direct the composition to specific target cells, namely cells that express integrin alpha10 beta1.

This amendment does not add new matter and is supported by the specification (see paragraphs 346-355, and in particular paragraphs 350-351). Paragraphs 346-355 of the specification disclose the usefulness of a monoclonal antibody of the invention, or fragment thereof, for targeting therapeutic agents to alpha10 beta1 expressing cells. This includes the targeting of therapeutic agents to alpha10 beta1 expressing cells by gene therapy. Paragraphs 350-355 specifically disclose various embodiments in which a monoclonal antibody of the invention, or fragment thereof, is used for targeting therapeutic genes to alpha10 beta1 expressing cells by gene therapy methods involving viral or non-viral delivery systems. Further relevant embodiments are disclosed in paragraphs 356-367 of the specification.

Applicant asserts that the Examiner's ground for rejection of claim 36 for indefiniteness is overcome by the described claim amendment and Applicant therefore respectfully requests that this rejection be withdrawn.

The amendment of claim 36 also resolves the Examiner's objection to Claim 37. Applicant respectfully requests that the rejection of claim 37 for indefiniteness also be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1-6 and 31-44 were rejected under 35 U.S.C. § 112, first paragraph, as not being enabled for lack of proof of ready public availability of the hybridoma DSM ACC2583 and NSO cells. The Examiner stated that a deposit of the hybridoma/cells under the Budapest Treaty may satisfy this enablement requirement. In response, Applicant states that the hybridoma cell line was deposited under the Budapest treaty at the DSMZ in Germany which acts as an International Depository Authority (IDA) for the deposit of biological material for the purposes of patenting under the Budapest Treaty. A copy of the confirmation by the DSMZ of the deposit under the Budapest treaty and of the viability of the deposited hybridoma cells is enclosed as evidence. Applicant respectfully requests that this rejection be withdrawn.

Claims 33-37 were rejected under 35 U.S.C. § 112, first paragraph, as not being enabled for the administration vehicles or pharmaceutical compositions referred to in these claims. The Examiner acknowledged, however, that the specification is enabling for a monoclonal antibody capable of binding to the extracellular I-domain of integrin alpha10, a hybridoma cell line, a composition and kit thereof. The rejection is based on the argument that undue experimentation is required to practice the claimed pharmaceutical composition/ administration vehicle with a reasonable expectation of success, because of (a) the absence of a specific and detailed description of use, (b) the absence of working examples providing evidence of reasonable expectation of

effectiveness *in vivo*, (c) the lack of predictability in the art at the time the invention was made, and (d) the absence of a rigorous correlation of pharmacological activity between the disclosed *in vitro* studies and an *in vivo* use. Applicant respectfully traverses.

Claims 33-37 pertain to pharmaceutical compositions or administration vehicles comprising a monoclonal antibody of the invention. These pharmaceutical compositions and administration vehicles are disclosed in the specification, for example, in paragraphs 346-356.

(a) Alleged absence of a specific and detailed description of use. The art at the time of filing described a large variety of antibodies against cell surface epitopes that have been successfully made and used *in vivo* in form of pharmaceutical compositions or administration vehicles. Most relevant, the art described a large variety of antibodies that had been developed as integrin antagonists and tested successfully in animal models or clinical trials. *Shimaoka M, Springer TA., Therapeutic antagonists and conformational regulation of integrin function, Nat Rev Drug Discov. 2003 Sep;2(9):703-16.* These included monoclonal antibodies directed against the I-domain of integrin alpha chains. As a case in point, phase III clinical trials for treatment of psoriasis had been successfully concluded using a monoclonal antibody against the I-domain of integrin alphaL. *Cather JC, Cather JC, Menter A., Modulating T cell responses for the treatment of psoriasis: a focus on efalizumab. Expert Opin Biol Ther. 2003 Apr;3(2):361-70.*

Therefore, one skilled in the art at the time of filing would have known how to make and use a pharmaceutical composition or administration vehicle comprising a monoclonal antibody of the invention. "Detailed procedures for making and using the

invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention." MPEP § 2164. Hence, a more specific and detailed description of use is not required to enable the invention.

(b) Alleged absence of working examples providing evidence of reasonable expectation of effectiveness *in vivo*. The fact that the specification does not contain a working example describing the use of a claimed pharmaceutical composition or administration vehicle *in vivo* does not by itself support a rejection based on non-enablement. "The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation." MPEP § 2164.02. In weighing the underlying factual findings in an enablement determination, the absence of a working example is not a strong factor if the art involved is predictable and well developed, as is the case here (see below).

(c) Alleged lack of predictability in the art at the time the invention was made. It was well established in the art at the time of filing that the I-domain is a major ligand-binding domain and that this domain recognizes the ligand directly. *Shimaoka M, Springer TA., Therapeutic antagonists and conformational regulation of integrin function, Nat Rev Drug Discov. 2003 Sep;2(9):703-16.* The art also recognized that because the I-domain binds the ligand directly, binding of another molecule, such as an inhibitory molecule, to the I-domain is "a straightforward way to block function [of the ligand-integrin interaction] competitively". *Id. at 713 (emphasis and parenthesis added).* Indeed, many of the inhibitory antibodies described in the art at that time were directed against the I-domain of integrins. *For example, Diamond, M.S., et al., The I domain is a*

major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J. Cell Biol. 120, 1031–1043 (1993); REF94. Hence, the art at the time of the invention was well developed and predictable, and there was a high degree of expectation of success *in vivo* for a pharmaceutical composition or administration vehicle comprising a monoclonal antibody that binds specifically to the I-domain of an integrin.

(d) Alleged absence of a rigorous correlation of pharmacological activity between the disclosed *in vitro* studies and an *in vivo* use. Finally, the alleged absence of rigorous correlation of pharmacological activity between disclosed *in vitro* studies and an *in vivo* use relates to the absence of a working example and the predictability in the art, as discussed above. "An applicant need not have actually reduced the invention to practice prior to filing." MPEP § 2164.02. Hence, the absence of actual *in vivo* data is not a strong factor, let alone a sufficient basis in itself, for rejecting claims that pertain to an *in vivo* use, in particular where the art at the time of the invention was well developed and predictable, as is the case here (see above).

Together -- considering the well developed state of the art, the manifold prior success of this type of invention, and the predictability of the art -- no undue experimentation would be required to practice the invention. Testing the pharmacology and efficacy of a pharmaceutical composition or administration vehicle comprising a monoclonal antibody against a cell surface epitope was standard procedure in the biotechnology or pharmaceutical industry at the time of the invention. The fact that such experimentation may involve animal models and clinical trials may make it complex, but

it does not make it undue, if the art typically engages in such experimentation. MPEP § 2164.01.

Based on the above considerations, Applicant respectfully request that the rejection of claims 33-37 as not being enabled be withdrawn.

Rejections under 35 U.S.C. § 102(b)

Claims 1-2 and 31, 33, 35-36 and 44 were rejected under 35 U.S.C. § 102(b) as being anticipated by WO 99/51639. Applicant respectfully notes that the second paragraph of section 13 ("...that the reference antibody does not specifically recognized by the monoclonal antibody produced by the deposited hybridoma...") was not understood.

Claims 1-2 and 44 have been cancelled, rendering the rejection of these claims moot.

Claims 31, 33, and 35-36 have been amended in response to this rejection by limiting the scope of the claims to pharmaceutical compositions and administration vehicles comprising specifically a monoclonal antibody or fragment thereof according to claim 5. Therefore, Applicant respectfully requests that the rejection of claims 31, 33, and 35-36 be withdrawn.

Rejections under 35 U.S.C. § 103(a)

Claims 1 and 3-4 were rejected under 35 U.S.C. § 103(a) as being unpatentable over WO99/51639 in view of *Owens et al.*. Claims 1 and 3-4 have been cancelled, rendering the rejection of these claims moot.

Claims 1 and 32, 38 and 40 were rejected under 35 U.S.C. § 103(a) as being unpatentable over WO99/51639 in view of US patent 6,096,873. Claim 1 has been

cancelled, rendering the rejection of this claim moot. Claims 38 has been amended in response to this rejection by limiting the scope of the claim to a kit comprising specifically a monoclonal antibody or fragment thereof according to claim 5. Furthermore, because of the amendments to claims 31 and 38, the scope of dependent claims 32 and 40 has also been narrowed to a claimed composition or kit comprising specifically a monoclonal antibody or fragment thereof according to claim 5. Therefore, Applicant respectfully requests that the rejection of claims 32, 38 and 40 be withdrawn.

Claim 39 was rejected under 35 U.S.C. § 103(a) as being unpatentable over WO99/51639 in view of *Male*. Because of the amendment to claim 38, the scope of the dependent claim 39 has now been narrowed to a kit comprising specifically a monoclonal antibody or fragment thereof according to claim 5. Thus, Applicant respectfully requests that the rejection of claim 39 is also withdrawn.

Provisional rejections for double patenting

Claims 1-2, 31, 33, 35-36 and 44 were provisionally rejected as being unpatentable over claims 135 and 137-143 of co-pending application 11/347,179 under the judicially created doctrine of non-statutory obviousness-type double patenting. Claims 1-2 and 44 have been canceled, rendering the rejection of these claims moot. The scope of claims 31, 33, and 35-36 has been narrowed by amendment to relate only to compositions and administration vehicles comprising specifically a monoclonal antibody or fragment thereof according to claim 5. Therefore, Applicant respectfully requests that these rejections be withdrawn.

Conclusion

As Applicant has addressed all of the Examiner's rejections and demonstrated that the now pending claims are patentable over the art of record, Applicant respectfully requests entry and timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: April 30, 2007

By: _____
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THERAPEUTIC ANTAGONISTS AND CONFORMATIONAL REGULATION OF INTEGRIN FUNCTION

Motomu Shimaoka and Timothy A. Springer

Integrins are a structurally elaborate family of adhesion molecules that transmit signals bi-directionally across the plasma membrane by undergoing large-scale structural rearrangements. By regulating cell–cell and cell–matrix contacts, integrins participate in a wide range of biological processes, including development, tissue repair, angiogenesis, inflammation and haemostasis. From a therapeutic standpoint, integrins are probably the most important class of cell-adhesion receptors. Recent progress in the development of integrin antagonists has resulted in their clinical application and has shed new light on integrin biology. On the basis of their mechanism of action, small-molecule integrin antagonists fall into three different classes. Each of these classes affect the equilibria that relate integrin conformational states, but in different ways.

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Members of the integrin family of adhesion molecules are non-covalently-associated α/β heterodimers that mediate cell–cell, cell–extracellular matrix and cell–pathogen interactions by binding to distinct, but often overlapping, combinations of ligands (BOX 1). Eighteen different integrin α-subunits and eight different β-subunits are present in vertebrates, which form at least twenty-four α/β heterodimers, perhaps making integrins the most structurally and functionally diverse family of cell-adhesion molecules[1,2] (FIG. 1). Half of integrin α-subunits contain inserted (I) domains, which are the principal ligand-binding domains when present[3,4]. The complexity, and structural and functional diversity of integrins allows this family of adhesion molecules to play a pivotal role in broad contexts of biology, including inflammation, innate and antigen-specific immunity, haemostasis, wound healing, tissue morphogenesis, and regulation of cell growth and differentiation[1,2]. Conversely, dysregulation of integrins is involved in the pathogenesis of many disease states, from autoimmunity to thrombotic vascular diseases to cancer metastasis[5]. Therefore, extensive efforts have been directed towards the discovery and development of integrin antagonists for clinical applications. Significant advances have been made in targeting αIIbβ3 on platelets for inhibiting thrombosis[6], αvβ3 and αvβ5 for blocking tumour metastasis, angiogenesis and bone resorption[7], and β2 integrins and α4 integrins on leukocytes for treating autoimmune diseases and other inflammatory disorders[8,9].

Small-molecule integrin inhibitors not only interfere with ligand binding, but also stabilize particular integrin conformations, which has provided insights into integrin structural rearrangements. In this review, we focus not only on antagonists of integrins as promising therapeutics, but also on what the drug discovery literature can teach us about the conformational regulation of integrin structure and function, and what structural and mutational studies on integrins teach us about the mechanism of action of antagonists. The reader is referred elsewhere for reviews of the extremely large variety of small molecules and antibodies that have been developed as integrin antagonists, and results in animal model studies and human clinical trials[5–17].

Overview of integrin structure

Integrin heterodimers and integrin domains. Integrins are large glycoproteins with multiple domains. Integrin ligand-binding function is tightly linked to molecular conformation. On activation, dramatic

Box 1 | **Integrin ligands**

Integrins bind extracellular matrix ligands as well as cell-surface receptors. Fibrinogen — a dimeric soluble plasma protein that plays important roles in blood clotting, wound healing and inflammation[106,107] — is also one of the most important extracellular matrix ligands for integrins including αIIbβ3, αvβ3, αMβ2 and αXβ2. Fibrinogen is proteolytically converted by thrombin to fibrin that self-assembles into an insoluble clot with a network-like structure. Binding to and crosslinking of αIIbβ3 on platelets by fibrinogen is required for platelet aggregation, which is crucial in thrombus formation. Fibrinogen also binds to αMβ2 and αXβ2, and is involved in inflammation by facilitating leukocyte adhesion to fibrinogen-deposited tissue and implants.

On the other hand, intercellular adhesion molecules (ICAMs) are immunoglobulin superfamily members that are among the most important cell-surface ligands for integrins including αLβ2, αMβ2, αXβ2 and αDβ2 (REF. 92). ICAM-1 is expressed on endothelial cells as well as on leukocytes. ICAM-1 is upregulated on endothelial cells by inflammatory stimuli such as tumour-necrosis factor-α. By interacting with β2 integrins, ICAM-1 plays a pivotal role in leukocyte migration into the tissue from the blood stream. ICAM-1 on antigen-presenting cells functions as a co-stimulatory molecule to T-cell receptors by signalling through leukocyte function-associated antigen-1 on T cells and taking part in immunological synapse formation[90].

rearrangements occur in the overall spatial relationships of integrin domains[4]. Understanding the structural basis of integrin activation in detail is essential for understanding the mechanism of antagonism by therapeutics, as well as for the design of second-generation antagonists with novel mechanisms of action.

Integrins comprise two non-covalently-associated type I transmembrane glycoprotein α- and β-subunits. Each subunit contains a large extracellular domain, a single transmembrane domain and, except for integrin β4, a short cytoplasmic tail (FIG. 2). The integrin α-subunit ectodomain of >940 residues contains four domains (five in I-domain-containing integrins) and the β-subunit of ~640 residues contains eight domains (FIG. 2). The crystal structure of extracellular parts of αvβ3 integrin that lacks an I domain revealed the structure in a bent conformation of eight domains, and a portion of the ninth, out of the twelve domains predicted to be present (FIG. 2b)[18]. The structures of INTEGRIN-EGF DOMAINS 2 and 3 of the β2 subunit were determined by nuclear magnetic resonance, and these complemented a part of the missing portions of the αvβ3 structure[19]. The N-terminal portions of the α- and β-subunits fold into the globular headpiece, which is connected through α- and β-tailpiece domains to the membrane[18,20–22]. Dramatic rearrangements occur in the orientation of these domains during integrin activation (FIG. 3a–c)[23]. In this review, we focus on the β-propeller, I-like and I domains, because these are the ligand-binding domains and the domains to which known antagonists bind (FIGS 3c and 4). However, it should be emphasized that in the bent conformation, extensive interfaces — totalling >4,000 Å^2 of solvent-accessible surface — are buried between the headpiece and tailpiece, and between the α-tailpiece and β-tailpiece. These interfaces stabilize the bent conformation, and are important in regulating the equilibrium between the bent and extended integrin conformations (FIG. 3a–c)[23,24].

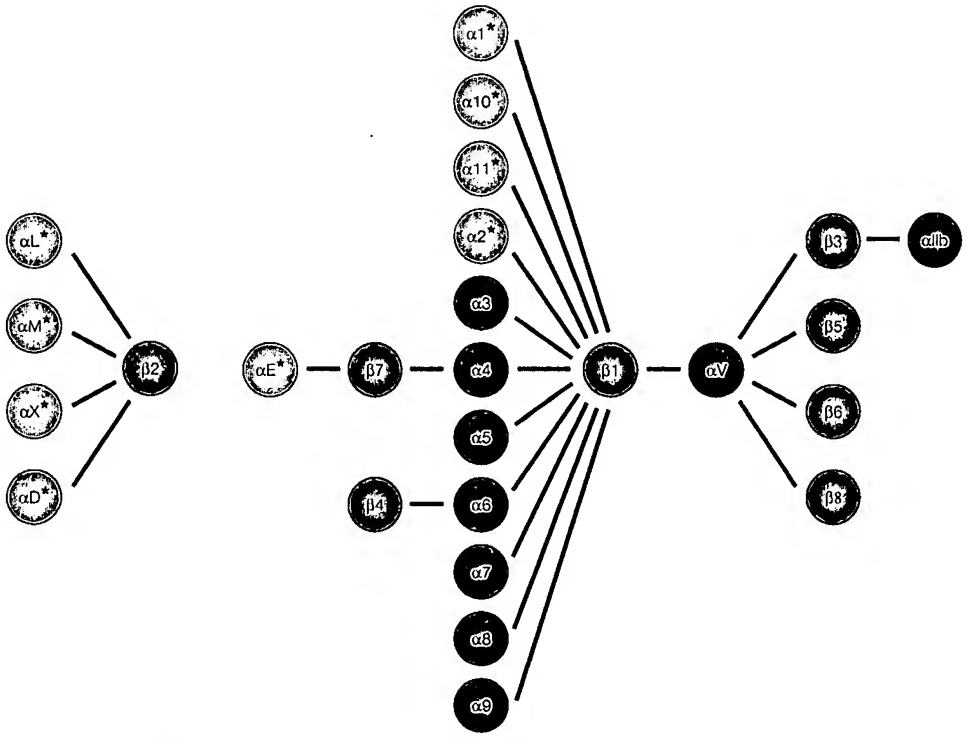

Figure 1 | **Integrin heterodimer composition.** Integrin α- and β-subunits form 24 heterodimers that recognize distinct but overlapping ligands. Half of the α-subunits contain I domains (asterisked).

INTEGRIN-EGF DOMAIN
A module in cysteine-rich repeats in the integrin β-subunit stalk region adopts a nosecone-shaped variant of the epidermal growth factor (EGF) fold, termed an integrin-EGF (I-EGF) domain.

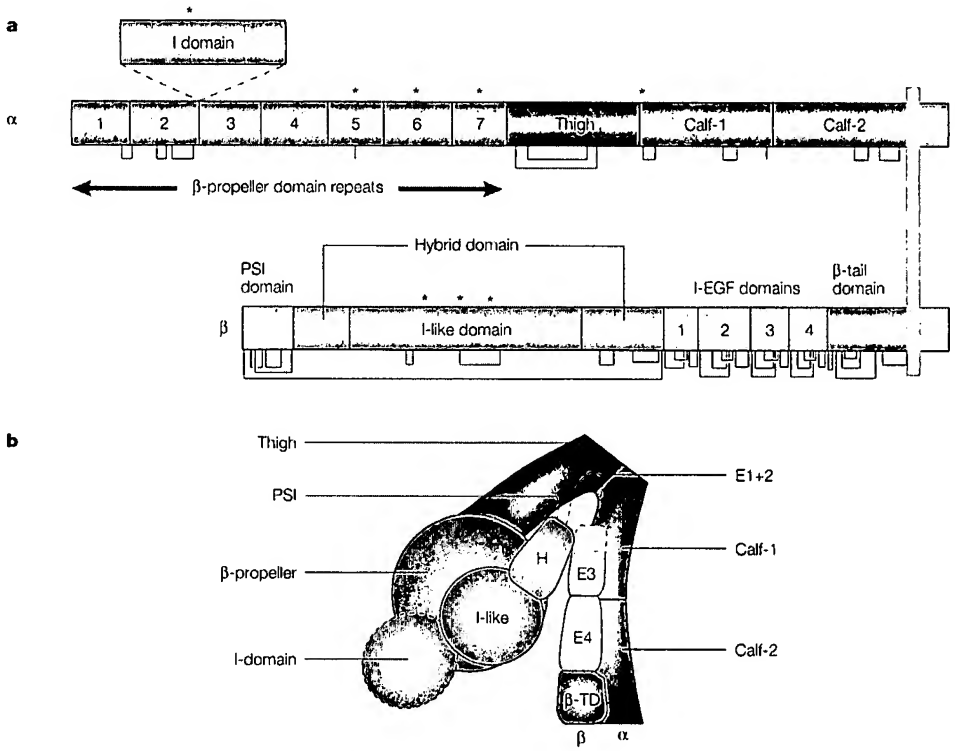

Figure 2 | **Integrin architecture. a** | Organization of domains within the primary structure. Some α-subunits contain an I domain inserted in the position denoted by the broken lines. Cysteines and disulphide bonds are shown as lines below the stick figures. Red and blue asterisks denote Ca^{2+}- and Mg^{2+}-binding sites, respectively. **b** | Arrangement of domains within the three-dimensional crystal structure of αvβ3 (REF. 18), with an I domain added. Each domain is colour-coded as in **a**. β-TD, β-tail domain; I-EGF, integrin-epidermal growth factor domain; PSI, plexin/semaphorin/integrin.

vWF-type A domains: I domain and I-like domain. All integrin β-subunits and half of integrin α-subunits contain von Willebrand factor (vWF)-type A domains of ~200 amino acids in length, also referred to as the I domain in the α-subunit and the I-like domain in the β-subunit, respectively (FIGS 1 and 2)[3,4]. Each domain adopts an α/β Rossmann fold with a metal-ion-dependent adhesion site (MIDAS) on the 'top' of the domain, whereas its C- and N-terminal connections are on the distal 'bottom' face[4,18,25,26] (FIGS 5 and 6). Divalent cations are universally required for integrins to bind ligands and the metal at the MIDAS directly coordinates to a glutamic acid (Glu) or aspartic acid (Asp) residue in the ligand. This metal-dependent interaction through the MIDAS has a central role in ligand recognition by the I and I-like domains.

I domain. The I domain, which is inserted between blades 2 and 3 of the β-propeller domain of the α-subunit (FIG. 2a)[27], is a major ligand-binding domain and recognizes ligand directly when it is present[28,29]. The ability of the I domain to bind ligand is controlled by conformational changes; the affinity of the I domain for its ligand is enhanced by downward axial displacement of its C-terminal helix, which is conformationally linked to alterations of the MIDAS loops and Mg^{2+} coordination[30–32] (FIG. 5). In the case of αLβ2, compared with the default, low-affinity conformation, downward displacements by one and two turns of the helix lead to intermediate- and high-affinity conformations with ~500- and 10,000-fold increased affinity, respectively[32].

Conversely, binding of ligand to the MIDAS of the I domain induces conformational change by stabilizing the high-affinity conformation. These changes include rearrangements in metal coordination in the MIDAS and backbone movements in the loops surrounding the MIDAS, which are linked to a downward axial displacement of the C-terminal helix (FIG. 4)[25,32,33].

I-like domain. The β-subunit I-like domain, which is inserted in the hybrid domain of the β-subunit (FIGS 2a, 3b and 3c), directly binds ligand in integrins that lack I domains in the α-subunit (FIGS 3c and 7). By contrast, when the I domain is present, the I-like domain functions indirectly by regulating the I domain (FIG. 4). Compared to the I domain, the I-like domain contains two long loops, including one which is important for determining ligand specificity, and is referred to as the specificity-determining loop (SDL)[34]. On either side of the MIDAS, the I-like domain contains

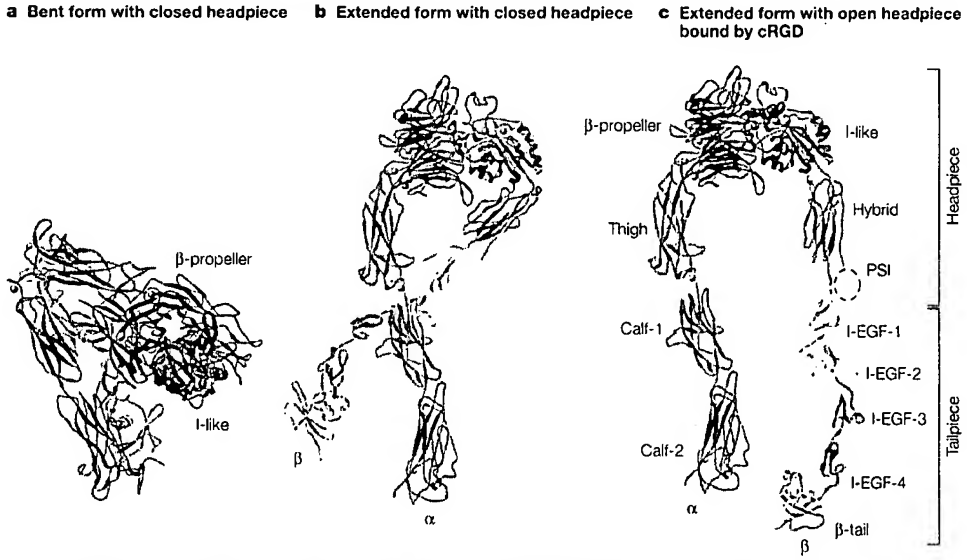

Figure 3 | **Domain organization and global conformational changes of the extracellular portion of integrins.** Three distinctive conformations are illustrated for integrin αvβ3, which lacks an I domain. The conformations are based on crystal[18,35], nuclear magnetic resonance[19] and electron microscopy[23] structures. Ribbon representations were prepared as described[108]. **a** | Bent conformation (low affinity). **b** | Extended conformation with closed headpiece (intermediate affinity). **c** | Extended conformation with open headpiece (high affinity) bound by cyclic Arg-Gly-Asp (cRGD) peptide (shown as a space-filling representation with black balls). Ca^{2+} and Mg^{2+} ions are gold and silver spheres, respectively. I-EGF, integrin-epidermal growth factor domain; PSI, plexin/semaphorin/integrin.

two adjacent metal coordination sites, the adjacent to MIDAS (ADMIDAS) site and the ligand-associated metal binding site (LIMBS) (FIG. 7)[18,35].

The function of the I-like domain seems to be regulated by conformational changes similar to those observed in the I domain, in which a downward movement of the C-terminal α-helix allosterically alters the geometry of the MIDAS and increases the affinity for ligand[4,36]. Outward swing of the hybrid domain relative to the I-like domain (FIGS 3b and c) is thought to be coupled to the downward shift of the C-terminal α-helix of the I-like domain[23,24].

β-propeller domain. The N-terminal region of the integrin α-subunit contains seven repeats of ~60 amino acids, which fold into a seven-bladed β-propeller domain[18,27] (FIG. 2). A β-propeller domain with the same topology is found in the trimeric G-protein β-subunit. The β-propeller domain directly participates in ligand recognition in those integrins that lack α I domains[4].

Structure of the headpiece in integrins that lack I domains. The structure of αvβ3 reveals that the I-like domain makes extensive contact with the β-propeller domain, with the 'top' ligand-binding faces of each domain oriented at ~90° to one another (FIG. 3c)[18]. Loops in blades 2, 3 and 4 of the β-propeller domain are prominent in the ligand-binding site. The structure of αvβ3 in complex with a cyclic peptide containing an Arg-Gly-Asp (RGD) sequence demonstrated binding to both the α- and β-subunits at the β-propeller/I-like domain interface (FIG. 3c). The Asp carboxylic acid side chain coordinates directly to the metal of the β-subunit I-like domain MIDAS, whereas the Arg side chain binds to the α-subunit β-propeller domain[35]. Mapping by mutagenesis of residues important in binding to fibrinogen, the biological ligand of αIIbβ3, demonstrated a much larger interaction surface, centered on blades 2 to 4 of the α-subunit β-propeller domain and the SDL loop of the β-subunit I-like domain (FIG. 7)[37,38].

Intriguing structural homology exists between the integrin β-propeller domain and the trimeric G-protein β-subunit, and between integrin I and I-like domains and G-protein α-subunits[18,27]. Dissociation of these domains on activation occurs in G-proteins; however, in integrin activation there is little rigid body movement of the β-propeller domain relative to the I-like domain[39]. Instead, ligand-binding affinity seems to be regulated primarily by conformational changes in loops of the I-like domain and β-propeller domain.

Structure of the headpiece in integrins that contain I domains. In integrins that contain I domains, the I domain binds ligand, whereas the β-propeller and I-like domains have a regulatory role (FIG. 4)[40,41]. The bottom of the I domain is connected at its N and C termini to blades 2 and 3 of the β-propeller, respectively. A linker of ~15 residues C-terminal to the I domain is located near the β-propeller/I-like domain interface, which corresponds to the ligand-binding face in integrins that lack I domains. It has been proposed that the I-like domain, when activated, interacts with a portion of this linker,

and thereby relays activation to the I domain[4,36,42]. The polypeptide linker between the C terminus of the I domain and β-sheet 3 of the β-propeller domain, including αL residue Glu-310, is important in I domain activation[41]. I domain activation is induced by a downward pull on the C-terminal α-helix or linker[4,43]. It is proposed that the universally conserved residue Glu-310 in the I domain linker is an 'intrinsic ligand,' and that binding of the activated β2 I-like domain to this intrinsic ligand pulls the C-terminal α-helix of the I domain downward, and activates high affinity for ligand (FIG. 4)[4,36,42].

Structural basis of signal transmission. Intracellular signalling pathways that are activated by other receptors (for example, receptors coupled to G-proteins or tyrosine kinases) impinge on integrin cytoplasmic domains and enhance the activity of the extracellular headpiece for ligand binding[44–46]. Recently, the basis for bi-directional signal transmission across the membrane by integrins has been explained. The integrin α- and β-cytoplasmic tails associate with each other and constrain the integrin in its inactive form. Dissociation of the α/β cytoplasmic tails by signals within the cell leads to the activation of the extracellular parts of the integrin[21,23,47–49]. In the latent, low-affinity state, the integrin assumes a bent conformation (FIG. 3a)[19,23]. Separation of the α- and β-subunit cytoplasmic domains is linked to separation of the transmembrane domains and the membrane-proximal segments of the α- and β-extracellular domains, which destabilizes the interface between the headpiece and tailpiece, and induces a switchblade-like opening to an extended conformation (FIG. 3b). This re-orientates the ligand-binding face and exposes activation epitopes in the tailpiece. In the extended conformation, two different conformations of the headpiece, termed closed (FIG. 3b) and open (FIG. 3c), are seen[23]. In the bent conformation, only the closed conformation of the headpiece is present[18]. Therefore, extension facilitates adoption of the open conformation of the headpiece, which corresponds to the ligand-bound and high-affinity conformation[23]. In the open headpiece conformation, there is a marked change in orientation between the I-like domain and hybrid domain (FIG. 3b and 3c), and this is postulated to be linked through movement of the C-terminal I-like domain α7 helix to conformational changes at the I-like domain MIDAS, similar to the conformational changes seen in I domains[23]. Linked changes in β-propeller loops may also occur. Notably, many antibody epitopes that are buried in the bent conformation become exposed in the extended conformation. As described below, ligand-mimetic integrin antagonists also induce the high-affinity, extended conformation. This has important clinical implications, especially for αIIbβ3 antagonists.

In solution, and apparently on the cell surface as well, integrins are not fixed in a particular conformation, but equilibrate between them[23] (FIG. 3a–c). Whether or not the equilibrium favours the bent, low-affinity conformation or the extended, high-affinity conformation is affected by the presence of activating

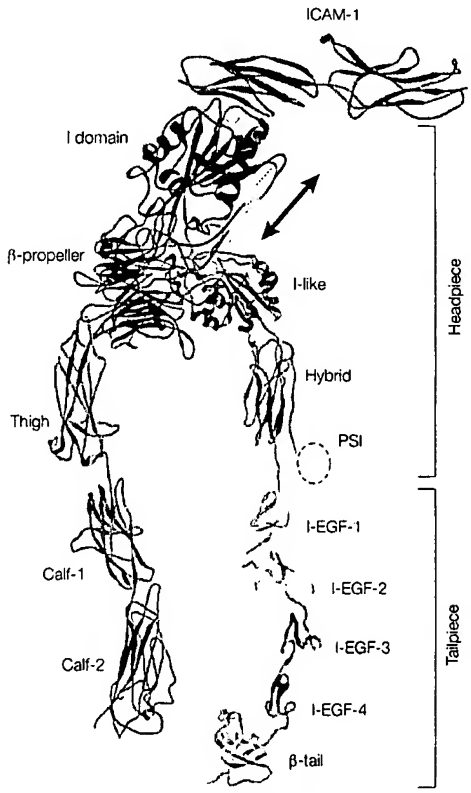

Figure 4 | **Model of integrin αLβ2, which contains an I domain, bound to ICAM-1.** Domains, except for the I domain and intercellular adhesion molecule-1 (ICAM-1), are the same as those of αvβ3 in FIG. 3c. The αL I domain and its complex with the N-terminal 2 domains of ICAM-1 are cartoons based on crystal structures[32]; the C-terminal I domain α-helix is represented by a cylinder in its low-affinity (blue) and high-affinity (orange) conformation. The I domain is joined at the point of its insertion in the β-propeller domain but its orientation is arbitrary; the I domain and ICAM-1 are shown at slightly larger scale for emphasis. Mg^{2+} ions at the metal-ion-dependent adhesion site of the I and the I-like domains are gold spheres. Ca^{2+} ions and a metal at the β2 ligand-associated metal binding site are not shown. I-EGF, integrin-epidermal growth factor domain; PSI, plexin/semaphorin/integrin.

intracellular factors and the concentration of extracellular ligands. Activation by signals within the cell (inside–out signalling) induces straightening and stabilizes the extended form. Binding of extracellular ligands also stabilizes the extended conformation and therefore enhances the separation of integrin tails, which transmits signals to the cytoplasm (outside–in signalling). Therefore, transition from the bent to the extended conformation is a bi-directional, allosteric mechanism for relaying conformational signals between the integrin headpiece and the cytoplasmic domains. All biological integrin ligands are multivalent, and can therefore also induce integrin clustering, which seems to be required, in addition to conformational change, for outside–in signalling.

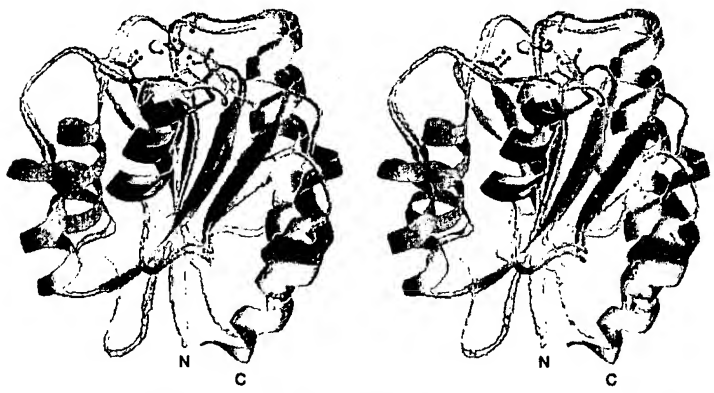

Figure 5 | **Stereo view of alternative conformations of the αM I domain.** The regions with significant conformational changes are shown in blue (closed, low affinity) and yellow (open, high affinity). Regions with similar backbones are in gray. Mg^{2+} ions and side chains of metal-coordinating residues at the metal-ion-dependent adhesion site are in blue (closed, low affinity) and yellow (open, high affinity) with red oxygen atoms. Ribbon diagrams are prepared as described[4].

Integrins lacking I domains: αIIbβ3

αIIbβ3 biology. αIIbβ3, which is the receptor for fibrinogen/fibrin, is expressed exclusively on platelets and their precursors, and mediates platelet aggregation and thrombus formation. On resting platelets, αIIbβ3 is inactive, but when platelets are exposed to agonists such as thrombin, ADP and platelet-activating factor, αIIbβ3 undergoes changes to the extended, active conformation that binds fibrinogen, and additional αIIbβ3 receptors are mobilized to the cell surface from an α-granule storage pool[10]. Platelet aggregation is a prerequisite for thrombus formation, which first diminishes and then stops blood supply to organs, and subsequently results in tissue ischaemia. Integrin αIIbβ3 has a key role in the pathogenesis of thrombotic cardiovascular diseases, such as ischaemic heart disease and stroke. The development of integrin antagonists was pioneered with αIIbβ3, for which there are three distinct clinically approved therapeutics and an extremely large medicinal chemistry literature[5,6,10].

Structural features of biological ligands. In addition to fibrinogen, αIIbβ3 binds to plasma and matrix proteins, such as vWF, fibronectin, thrombospondin and vitronectin. These ligands all share an RGD or RGD-like motif that is central in their receptor binding site. A subset of integrins that lack I domains, including αIIbβ3, αvβ3, and α5β1, recognize the RGD motif. Plasma fibrinogen is a disulphide-linked dimer, with each monomer containing disulphide-linked α-, β-, and γ-subunits. The monomers dimerize at their N-terminal ends. The crucial recognition motif for αIIbβ3 is an RGD-like Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) motif near the C-terminal end of the γ-subunit, although two RGD sites in the α-subunit may also be recognized[3].

A family of integrin ligands containing RGD or KGD motifs found in snake venom are named disintegrins for their ability to inhibit platelet aggregation by interacting with αIIbβ3. In contrast to fibrinogen, which binds only active αIIbβ3, the disintegrin echistatin can bind potently to both active and inactive forms of the integrin[50].

Antagonist action and effects on conformation. Using the RGD motif and disintegrins as leads, αIIbβ3 antagonists have been developed that are cyclic peptides containing the RGD or KGD sequence, peptidomimetics, or small molecules that imitate the integrin-binding properties of the RGD peptide in terms of overall geometry and the presence of a basic moiety and free carboxyl group[6] (FIG. 8a). The antagonists function as ligand-mimetic competitive inhibitors. A crystal structure of a cyclic RGD peptide bound to the closely related αvβ3 integrin demonstrates that the Arg guanidinium moiety forms salt bridges to Asp residues in β-propeller loops, and the Asp carboxyl group ligates the metal ion in the I-like domain MIDAS, whereas the Gly makes few interactions and essentially serves as a spacer[35]. These observations are in excellent agreement with structure–activity relationships observed for small-molecule antagonists[6,51]. Furthermore, the contacts of the cyclic RGD peptide with αvβ3 (FIG. 3c) correspond to the centre of the binding site for fibrinogen in αIIbβ3 defined by mutagenesis (FIG. 7).

Antagonist-induced active conformation. Binding of natural ligands to integrins induces exposure of neoepitopes referred to as ligand-induced binding site (LIBS) epitopes[20,52–55]. The exposure of LIBS epitopes is a consequence of conformational changes, and is usually induced by activation as well as binding of ligands. Some, if not all, small-molecule antagonists of αIIbβ3 induce LIBS epitopes just as natural ligands do, indicating that the antagonists induce the active conformation (FIG. 9a–c)[56–58].

Two-step binding kinetics also demonstrate that a conformational rearrangement occurs on ligand binding. Two-step kinetics have been demonstrated for both the binding of purified fibrinogen to purified integrin αIIbβ3 (REFS 59,60), and the binding of fluorescent RGD peptidomimetics to αIIbβ3 on intact platelets[61]. In a fast reaction, a low-affinity complex is first formed. In a reaction with slower kinetics, the low-affinity complex is converted to a higher-affinity complex. The $t_{1/2}$ for conversion by peptidomimetics is 11–16 seconds (REF. 61). These findings are consistent with observations that cyclic RGD peptides bind to the bent, low-affinity conformation of αvβ3 (REF. 35) (FIG. 3a), and induce conversion to the extended conformation with open headpiece[23] (FIG. 3c).

Most ligand-mimetic antagonists induce LIBS epitopes that are topographically widely distributed over the αIIb and β3 subunits, whereas some, such as lamifiban (Ro44-9883) and tirofiban (MK383), induce only a subset of LIBS[58,62]. Some antagonists, including tirofiban and lamifiban, bind to the active form of αIIbβ3 with markedly higher affinity than to the inactive form, similarly to the natural ligand fibrinogen. Others, such as L-736622, bind to active and inactive forms with equal affinity, similarly to the disintegrin echistatin[50].

In addition to the induction of the active conformation, high-affinity small-molecule antagonists of αIIbβ3 tighten association between the α- and β-subunits. When αIIbβ3 is incubated in SDS at room temperature, the non-covalently-associated subunits separate and migrate as α- and β-monomers in SDS–PAGE. However, when pre-treated with high-affinity small-molecule antagonists or echistatin, αIIbβ3 migrates as an α/β complex in SDS–PAGE[63–65]. Formation of the SDS-stable α/β complex requires cations, possibly because of the metal requirement for antagonist binding to the I-like domain MIDAS. A fragment of αIIbβ3 containing only headpiece domains is also stabilized as a dimer in SDS–PAGE by a high-affinity antagonist[19]. This shows that it is the α-subunit β-propeller interface with the β-subunit I-like domain that is stabilized by the compounds, which is consistent with compound binding across this interface.

All clinically approved αIIbβ3 antagonists elicit THROMBOCYTOPAENIA in a small subgroup of up to 2% of patients[6,10,66]. The exposure of neoepitopes by ligand-mimetic antagonists is an obvious concern, although the antibody antagonist abciximab, which does not induce neoepitopes, also elicits thrombocytopaenia. Recently, drug-dependent antibodies against αIIbβ3 have been associated with the incidence of thrombocytopaenia in trials of the oral αIIbβ3 antagonist roxifiban[10,66]. Interestingly, the antibodies show specificity for the αIIbβ3–antagonist complex; that is, a lack of, or limited, crossreactivity with αIIbβ3 complexed with chemically distinct antagonists. Furthermore, drug-dependent antibodies were found to pre-exist in the general population at a frequency of 1%[66].

Agonistic effects of antagonists. The functional consequences of the antagonist-induced active conformation have been hotly debated[6,67,68]. Agonistic effects of antagonists have been demonstrated with RGD peptides and small-molecule antagonists that are known to induce LIBS[56,57]. Purified αIIbβ3, pre-incubated with RGD peptide, followed by removal of bound peptide, is activated for binding to soluble fibrinogen, whereas the continued presence of RGD peptide inhibits fibrinogen binding. When platelets are incubated with RGD or small-molecule antagonists, then fixed, and washed to remove the antagonists, fibrinogen binding is activated. So, antagonists not only expose LIBS epitopes but also induce the high-affinity ligand-binding conformation. By contrast, on intact, unfixed platelets, the antagonist-induced active conformation is reversible and does not persist after the antagonist is washed out[56,68]. The reversibility of the antagonist-induced active conformation in intact platelets has important implications for the development of therapeutics, because without it drug clearance would be expected to result in enhanced platelet aggregation. Membrane and/or cytoplasmic interactions have been proposed to explain the reversal in the antagonist-induced active conformation that is seen in intact platelets but not in purified αIIbβ3 (REF. 57).

SDS–PAGE
(Sodium dodecyl sulphate–polyacrylamide gel electrophoresis). A method for resolving a protein into its subunits and determining their separate molecular weights.

THROMBOCYTOPAENIA
A disorder in which the number of platelets is abnormally low, and which is sometimes associated with abnormal bleeding.

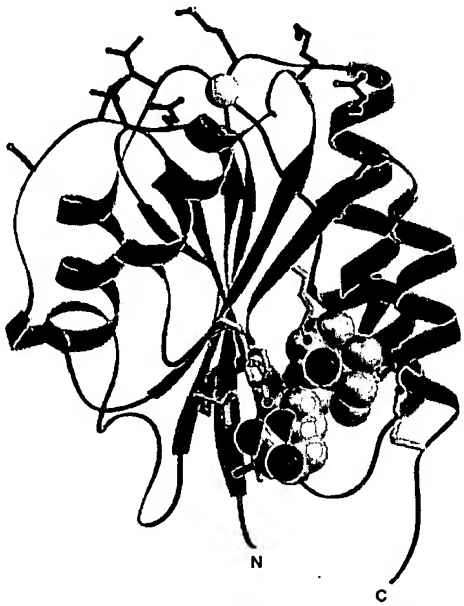

Figure 6 | **Ribbon diagram of the αL I domain in complex with an α I allosteric antagonist[93].** The I domain adopts a Rossmann fold in which the central β-strands (green) are surrounded by seven α-helices (blue). A Mg^{2+} ion (yellow) is located at the metal-ion-dependent adhesion site (MIDAS) on the 'top' of the domain, whereas the C and the N termini, which connect to the β-propeller domain, are on the bottom. The α I allosteric antagonist (shown by a space-filling representation with silver carbon atoms and red oxygen atoms) binds in the hydrophobic pocket underneath the C-terminal α-helix and stabilizes the I domain in the closed conformation. The side chains within the antagonist-binding pocket are shown with gold bonds and carbon atoms, red oxygen atoms, and blue nitrogen atoms. The residues crucial for binding to ICAM-1 and ICAM-2 are shown with purple side chains and yellow sulphur, red oxygen, and blue nitrogen atoms. Note that these residues are located around the MIDAS, distal from the antagonist binding site.

A striking feature of integrins is their ability to transmit signals bi-directionally across membranes, and so the induction of an active conformation by antagonists raises the question of whether they could trigger outside–in signalling in a manner similar to natural ligands. However, antagonists fail to trigger Ca^{2+} signalling in resting platelets, although after pre-activation by thrombin, antagonists could elicit Ca^{2+} signalling indirectly via thromboxane A_2 production[58]. Antagonists did not induce degranulation as measured by P-selectin expression in resting platelets[69]. In contrast to biological ligands, αIIbβ3 antagonists are monovalent, and this seems to explain their lack of efficacy in initiating outside–in signalling in spite of potent induction of LIBS. Indeed, RGD peptides immobilized on beads, but not in solution, elicit Ca^{2+} signalling through αvβ3 integrin[70].

Integrins lacking I domains: αvβ3

αvβ3 biology. The integrin αvβ3 recognizes a wide range of extracellular matrix ligands, including vitronectin, fibrinogen, fibronectin, thrombospondin,

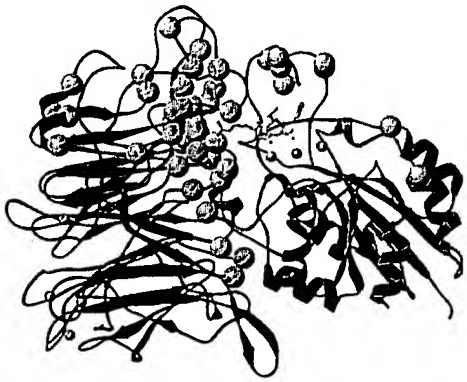

Figure 7 | **αIIbβ3 headpiece and critical residues for ligand binding.** The αIIb β-propeller domain is modelled on that of αvβ3. The β-propeller (blue) and the I-like domain (red) are shown as ribbon representations. Critical residues for binding of αIIbβ3 to fibrinogen were identified by mutagenesis and defined as described[37,38] and are shown as Cα-spheres of similar colour to the ribbon backbone. Ca^{2+} ions at the β-propeller and the adjacent to metal-ion-dependent adhesion site of the I-like domain are orange. Mg^{2+} ions at the metal-ion-dependent adhesion site and ligand-associated metal binding site are black and silver, respectively. A cyclic Arg-Gly-Asp (RGD) peptide is shown with yellow bonds and red oxygen atoms and blue nitrogen atoms.

vWF and osteopontin. Cells including smooth muscle cells, endothelial cells, monocytes and platelets express αvβ3. Increased expression of αvβ3 is observed on the vasculature in tumours and on several invasive malignant cells, indicating a role in tumour angiogenesis and metastasis[71]. Inhibition of angiogenesis and tumour cell growth by αvβ3 and αvβ5 antagonists has been seen *in vivo*[7,71], but, surprisingly, genetic ablation of these receptors does not block angiogenesis and can even enhance it[72]. Among other mononuclear phagocytes, αvβ3 is expressed on osteoclasts, where it has an important function in interacting with the bone matrix and in bone resorption. Antagonists of αvβ3 block bone resorption *in vitro* and *in vivo*, making αvβ3 an important target in diseases such as osteoporosis[73].

Antagonist action and effects on conformation. Antagonists of αvβ3 (FIG. 8a) are RGD mimetics that were developed to be selective for αvβ3 as compared with αIIbβ3, and compared with integrins containing the αv subunit associated with other integrin β-subunits (αvβ1, αvβ5, αvβ6 and αvβ8; FIG. 1). The specificity of cyclic-RGD-containing peptides for αvβ3 over αIIbβ3 was achieved by chemically favouring a type I β-turn over a type II β-turn[74]. This alteration of the β-turn changes the orientation of the Arg residue that binds to the α-subunit relative to the Asp residue that binds to the β-subunit, thereby affecting α-subunit specificity. Similarly, non-peptidic antagonists that are selective for αIIbβ3 can be converted to selectivity for αvβ3 by chemically altering the structure and orientation of the basic moiety that mimics the Arg guanidinium group[51]. In agreement with this finding, the salt bridges that αv and αIIb β-propeller domains form with the Arg guanidinium group must differ. In the αvβ3–cyclic RGD crystal structure, Asp residues in the loops connecting β-propeller blades 2 and 3, and connecting blades 3 and 4, donate salt bridges[35]. In each of these loops in αIIb, an Asp is not present in the same position, but an Asp residue is present one residue further towards the C terminus in the same loop.

As described above, αvβ3 antagonists bind to the bent, low-affinity conformation of αvβ3 (REF. 35) and induce conversion to the extended, high-affinity conformation[23] and exposure of LIBS epitopes[75,76]. High-affinity αvβ3 antagonists stabilize association of the αv and β3 subunits in SDS[65]. An interesting agonist activity of a cyclic RGD antagonist has been observed[77]. At concentrations near the K_D of the antagonist, it stimulated binding of purified αvβ3 to multiple ligands, whereas at higher concentrations it inhibited binding; however, a similar agonistic effect on native cell-surface αvβ3 has not been reported.

Integrins lacking I domains: α4β1

α4β1 biology. The integrin α4β1 is expressed on lymphocytes and monocytes, as well as on some connective tissue cells. There is a transient requirement for α4 in placentation and early heart development; however, in adult animals α4 is much more important in immune function[78]. α4β1 also functions in interactions between bone marrow stromal cells and haematopoietic progenitor cells, particularly B-cell progenitors. α4β1 is important in physiological lymphocyte trafficking, as well as in leukocyte migration during inflammation, and is implicated in the pathogenesis of autoimmune and allergic diseases[4,79]. Furthermore, a monoclonal antibody to α4 has shown therapeutic benefit in controlled trials in multiple sclerosis and Crohn's disease patients[80,81]. α4β1 binds to the immunoglobulin superfamily (IgSF) molecule vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelial cells, and mediates both rolling and firm adhesion in shear flow[2,82]. α4β1 also binds to fibronectin in the extracellular matrix.

Structural features of biological ligands. In contrast to other integrins, including αIIbβ3, αvβ3, and α5β1, which bind to the RGD motif in the tenth fibronectin type 3 (FN3) module of fibronectin, α4β1 binds to a distinct site. α4β1 binds to an LDV motif in fibronectin which is present in an alternatively spliced region following the fourteenth FN3 module. This region is termed connecting segment 1 (CS-1), and is non-homologous to the fibronectin type 1, type 2 and type 3 modules.

VCAM-1 contains six tandem IgSF domains. α4β1 recognizes both domains 1 and 2. The most crucial site for recognition is an Asp residue in an IDS motif that protrudes from domain 1 near the interface with domain 2. The three-dimensional structure of domains 1 and 2 of VCAM-1 shows that the short loop bearing the IDS sequence is highly ordered[83], in contrast to the flexible, long loop bearing the RGD sequence in FN3 module 10 of fibronectin[84]; a structure for the CS-1 segment of fibronectin is not yet available.

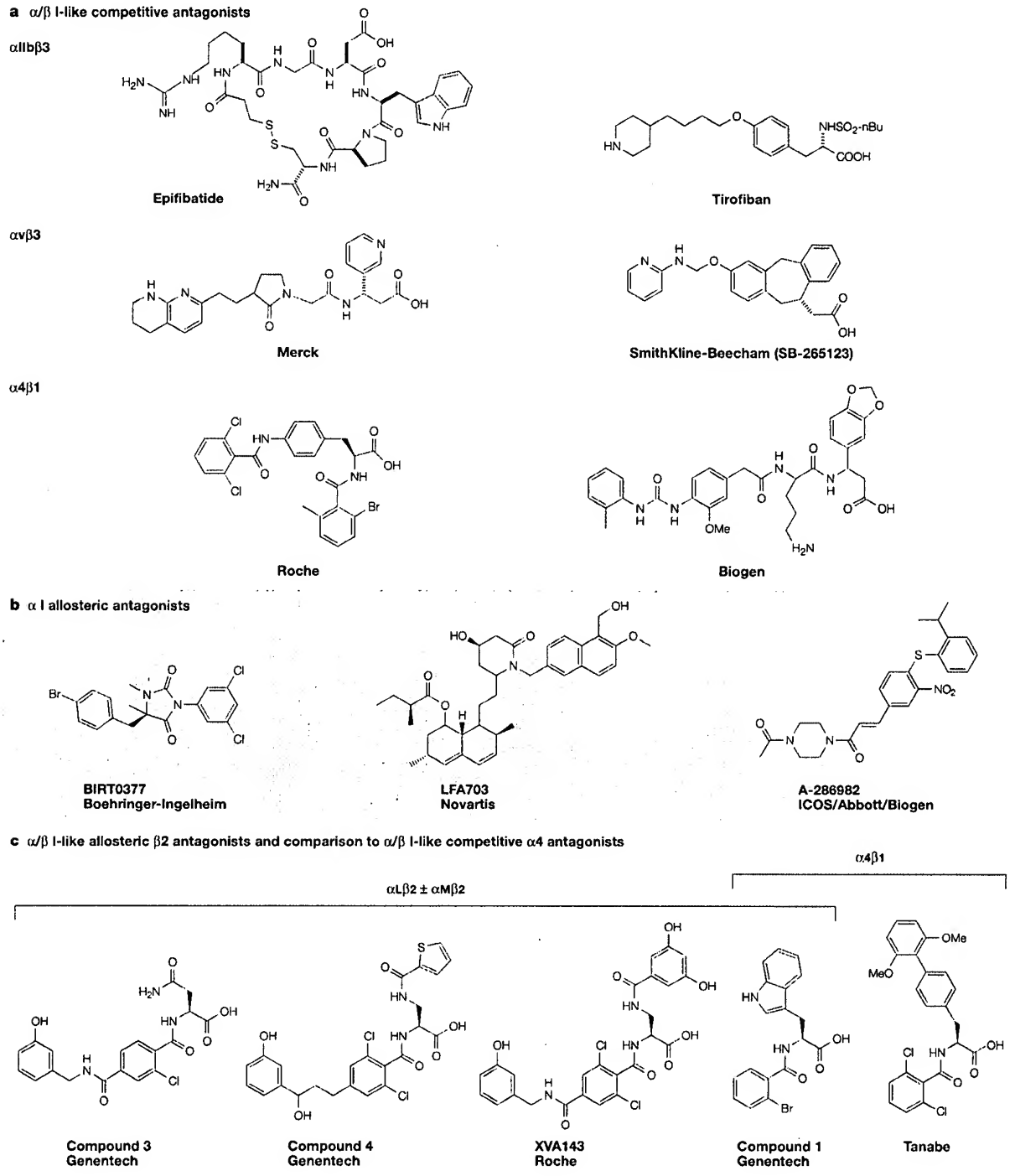

Figure 8 | **Chemical structures of small-molecule integrin antagonists. a** | α/β I-like competitive antagonists. Two clinically approved small-molecule antagonists of αIIbβ3 (REF. 6), two representative αvβ3 antagonists[109,110], and two representative α4β1 antagonists[14] are shown. **b** | α I allosteric antagonists. BIRT0377 (REF. 96), LFA703 (REF. 111), and A-286982 (REF. 97) are shown. **c** | α/β I-like allosteric β2 antagonists compared with α/β I-like competitive α4 antagonists. The antagonists are all poly-substituted (*S*)-2-benzoylaminopropionic acids. Compounds 1, 3, 4 (REFS 102,103), XVA143 (REFS 98,101), and a Tanabe α4β1 antagonist[14] are shown. The integrins antagonized by these compounds, which both contain I domains (αLβ2 and αMβ2) and lack I domains (α4β1) are indicated.

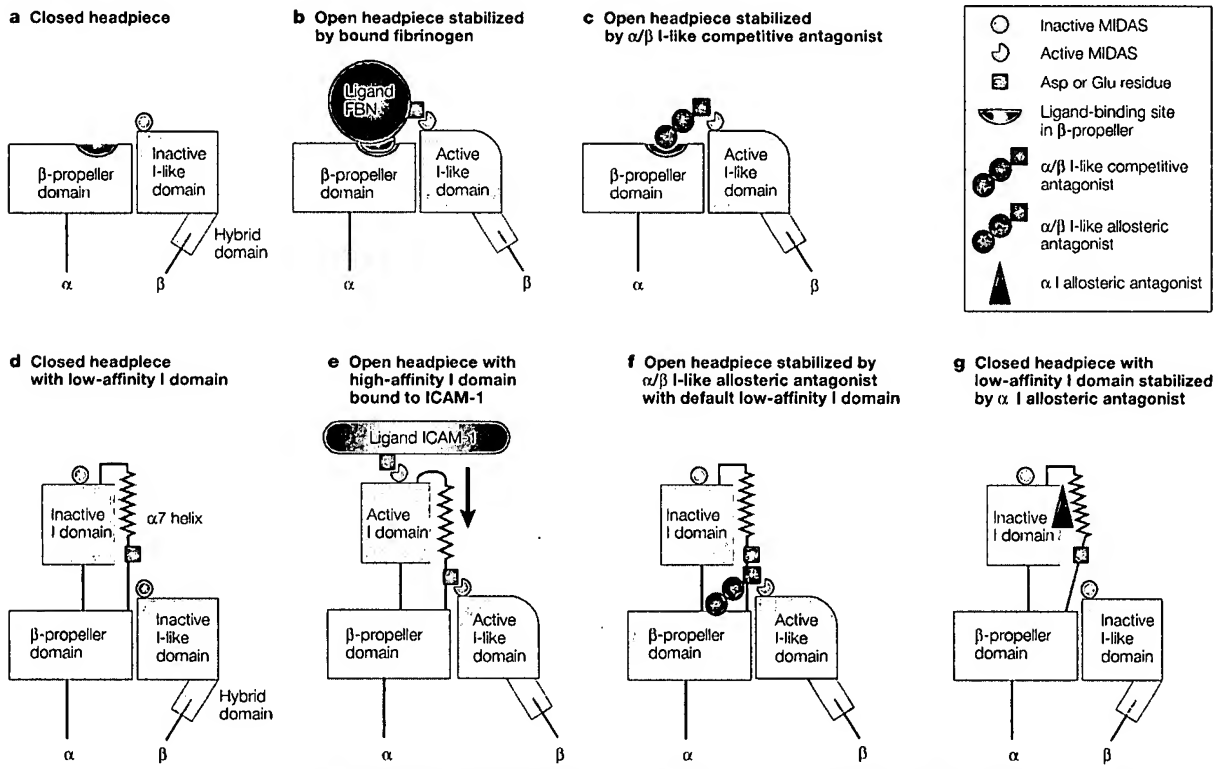

Figure 9 | **Mechanisms of inhibition and impact on integrin conformation of small-molecule antagonists.** Cartoons illustrate conformational changes in the headpiece of a representative integrin that lacks an I domain, αIIbβ3 (**a–c**), and a representative integrin that contains an I domain, αLβ2 (**d–g**). For clarity, only headpiece domains are shown, and domains in legs are simplified as lines. The outward-oriented (obtuse angled) hybrid domain correlates with exposure of activation-dependent epitopes in the legs and adoption of the extended conformation. FBN, fibrinogen; ICAM-1, intercellular adhesion molecule-1; MIDAS, metal-ion-dependent adhesion site.

Antagonist action and effects on conformation. Starting from cyclic peptides or random screening, a wide variety of small-molecule α4β1 antagonists have been developed, all of which contain a crucial carboxyl group that mimics the Asp of the LDV or IDS sequences[14,15,17] (FIG. 8a). Chemical crosslinking demonstrated binding of one of these antagonists to the MIDAS loops of the β1 I-like domain, which confirms binding to the same site as biological ligands[85]. Mutagenesis has demonstrated the importance of the β1 I-like domain MIDAS and nearby α4 β-propeller loops in the binding of fibronectin and VCAM-1 (REF. 86). An LDV peptide that blocks α4β1-mediated adhesion induces LIBS epitopes[54,87]. Therefore, ligand mimetics seem to stabilize α4β1 integrin in an active conformation. Multiple affinity states of α4β1 are revealed by binding of a fluorescent LDV peptide derivative[88].

Integrins containing I domains: β2 integrins

Biology of β2 integrins. The β2 integrins (αLβ2 or lymphocyte function-associated protein-1; αMβ2 or Mac-1; αXβ2 or p150,95; and αDβ2) (FIG. 1) are expressed exclusively on leukocytes. αLβ2 and αMβ2 are crucial in leukocyte migration into sites of inflammation, and, in the case of αLβ2, into lymphoid tissues (BOX 2) (REFS 2,89). αLβ2 is required for a wide variety of cell–cell interactions, including T cells with antigen-presenting cells, B cells with T cells, and natural killer cells with target cells, and acts as a co-stimulatory molecule in essentially all T-cell responses, which correlates with its participation in the formation of the IMMUNOLOGICAL SYNAPSE[90]. As shown by mutation of the β2 integrin subunit in leukocyte adhesion deficiency I, β2 integrins are essential for host defence against microorganisms. Furthermore, β2 integrins are important in the pathogenesis of leukocyte-mediated tissue injuries in inflammation and autoimmunity[13]. Of the leukocyte integrins, only αLβ2 is expressed on most lymphocytes, whereas αMβ2 predominates on neutrophils (αLβ2 and αXβ2 are also present). Diseases in which neutrophils are important, such as cerebral and myocardial infarction and shock, have usually been targeted with antagonists of all β2 integrins or of αMβ2. By contrast, diseases in which lymphocytes are important, such as psoriasis, rheumatoid arthritis, and organ transplant rejection, have usually been targeted with antagonists specific for αLβ2 (REFS 5,12,13,91). Phase III clinical trials in psoriasis with a monoclonal antibody against the αL I domain have been successfully concluded[11].

IMMUNOLOGICAL SYNAPSE
T-cell recognition of an antigen presenting cell (APC), which is the initial and crucial process in the antigen-specific immune response, takes place at the nanometer-scale gap of the interface between the T cell and APC. This interface is a specialized cell–cell junction, at which crucial signals to initiate and maintain the immune response are transduced from APC to T cell or vice versa. The interface is called an immunological synapse after the neuronal synapse, a segregated gap through which information is transmitted in chemical form (neurotransmitter) from one neuron to another.

Structural features of ligands for β2 integrins. αLβ2 binds exclusively to IgSF cell-surface molecules termed intercellular adhesion molecules (ICAMs), whereas αMβ2 binds to ICAM-1, fibrinogen, complement component iC3b and many other protein ligands. The major endothelial ligand for both αLβ2 and αMβ2 is ICAM-1, which comprises five tandem Ig-like domains[92]. The binding sites for αLβ2 and αMβ2 are located in domain 1 and domain 3 of ICAM-1, respectively. An acidic residue, Glu-34, in domain 1 of ICAM-1 directly ligates the metal at the MIDAS motif of the αL I domain, as recently shown by X-ray crystallography[32] (FIG. 4). In contrast to the crucial Asp in VCAM-1, which is in a protruding loop, the crucial Glu of ICAM-1 is present in a β-sheet in a relatively flat surface[93]. A different acidic residue in domain 3 is implicated in recognition by the αM I domain[93].

Antagonist action and effects on conformation

Direct inhibition of ligand binding to the I domain. As the top face of the I domain around the MIDAS directly binds ligands, binding of inhibitors to the same site on the I domain is a straightforward way to block function competitively. Indeed, many inhibitory antibodies map to the I domain[28,94]. Although most inhibitory I domain antibodies directly block ligand binding, some that bind more distal to the MIDAS inhibit ligand binding indirectly, similarly to the α I allosteric antagonists discussed below[41].

Allosteric I domain inhibitors. As discussed above, the affinity of the I domain is allosterically regulated by conformational change. Downward axial displacement of the C-terminal helix is linked to structural rearrangements at the ligand-binding site, the MIDAS (FIG. 5 and 9d,e). One class of small-molecule inhibitors, termed α I allosteric antagonists (FIG. 8b), bind underneath the C-terminal α-helix of the αL I domain (FIG. 6) (REFS 95–97). The antagonists bind to and stabilize the closed conformation of the I domain, inhibit conversion to the high-affinity, open conformation, and thereby allosterically inhibit ligand binding to the distal MIDAS site (FIG. 9g). The mode of action of the antagonists is confirmed by a mutant αL I domain that is locked in the open, high-affinity conformation with an engineered disulphide bond that stabilizes the position of the C-terminal α-helix (the high-affinity I domain)[30,40,41]. αLβ2 containing the locked open, high-affinity I domain is resistant to inhibition by α I allosteric antagonists[41].

Binding of α I allosteric antagonists to the pocket under the C-terminal α-helix of the I domain affects not only the conformation of the I domain regionally, but also the conformation of the αLβ2 heterodimer globally. Some α I allosteric antagonists perturb the binding of antibodies against the I-like domain[98]. Furthermore, the antagonists reduce exposure by the activating agent Mn^{2+} of activation-dependent epitopes in the β2 I-like domain, as well as in the α- and β-subunit legs[99,100]. Downward movement of the I domain C-terminal helix seems to be a prerequisite for binding of the intrinsic ligand Glu-310 in the αL I domain linker to the MIDAS of the β2 I-like domain (FIG. 9). In the absence of this interaction, activation of the I-like domain is inhibited, as shown by suppression of exposure by Mn^{2+} of an epitope in the I-like domain. Suppression of epitope exposure in the α- and β-legs shows that the α I allosteric antagonists shift the conformational equilibrium toward the bent conformation. Therefore, the conformation of the I domain is linked, apparently through the I-like domain, to the conformation of the leg domains. These results highlight the extensiveness of conformational linkages within integrins.

α/β I-like allosteric antagonists. The I-like domain of the β2 subunit of αLβ2 is a regulatory domain[41]. A class of αLβ2 and αMβ2 small-molecule antagonists patented by Roche and Genentech[101,102] (FIG. 8c) has recently been found to perturb the interface between the I domain and the I-like domain[98,100]. These inhibitors are polysubstituted (*S*)-2-benzoylaminopropionic acids, in common with some inhibitors of α4β1, which lacks an I domain (FIG. 8). Indeed, compound 1, which was used as a lead to develop αLβ2 and αMβ2 antagonists at both Genentech and Roche, was initially discovered during random screening as an α4β1 antagonist at Roche, and inhibits

Box 2 | **Multi-step model of leukocyte migration**

Infiltration and accumulation of leukocytes in the extravascular space is a hallmark of inflammation. Leukocyte–endothelial cell interaction, which involves multiple processes mediated by adhesion molecules and chemokines, is crucial in guiding leukocytes from the blood stream to the site of inflammation[2]. The initial step is rolling, which is mediated by selectins and their carbohydrate ligands and, additionally, α4 integrins. The rapid k_{on} and k_{off} of selectin–carbohydrate ligand interaction allows flowing leukocytes to tether and roll along endothelial cells under shear flow. Rolling slows down flowing leukocytes and places them in proximity to endothelial cells where chemokines are transported and expressed. Rolling facilitates encounter of leukocytes that express chemokine receptors to the corresponding chemokines presented on the apical surface of the endothelial cells. The second step is activation of G-protein-coupled receptors on leukocytes by the chemokine, which triggers signals that activate integrins. In turn, the ligand-binding activity of integrins is rapidly enhanced by inside–out signalling. Binding of integrins to ligands on endothelium such as intercellular adhesion molecule-1 mediate firm adhesion and arrest of the leukocyte on the endothelium. These three steps take place in sequence but not in parallel. So, inhibition of any one of the three steps results in essentially complete blockade of leukocyte migration. Although the β2 integrins, αMβ2 and αLβ2, are most generally utilized in neutrophil and lymphocyte migration, respectively, α4 integrins such as α4β1 and α4β7 also participate in leukocyte migration, and play a pivotal role in certain tissues. The α4β1 and α4β7 integrins mediate rolling adhesion prior to activation, and firm adhesion after activation.

αLβ2 and α4β1 with similar micromolar potency. This novel class of αLβ2 antagonists cannot inhibit the binding of isolated wild-type or mutant intermediate-affinity or high-affinity I domains to ICAM-1, whereas purified ICAM-1 does inhibit these binding interactions[100]. Therefore, the Genentech compounds do not mimic ICAM-1, as previously suggested[103]. Furthermore, these inhibitors bind to αLβ2, even when the α I domain is deleted, but do not bind when the I-like domain MIDAS is mutated[100]. They can demonstrate α-subunit selectivity, indicating that a portion of the α-subunit near the I-like domain — probably the β-propeller domain — might be involved in binding. Therefore, they are designated as α/β I-like allosteric antagonists.

As described above, the α I domain seems to be activated when the β I-like domain binds to a conserved Glu residue in the linker following the I domain; that is, the intrinsic ligand. The findings indicate that α/β I-like allosteric antagonists bind to the MIDAS of the β2 I-like domain as mimics of the intrinsic ligand, competitively inhibit binding of the intrinsic ligand in the I domain linker to the I-like domain, and consequently leave the I domain in the low-energy, inactive, closed conformation (FIG. 9f). At the same time, the α/β I-like allosteric antagonists stabilize the I-like domain in its active configuration by mimicking intrinsic ligand binding, as shown by induction of activation epitopes in the β2 I-like domain and αL and β2 legs[100]. So, as a consequence of I-like domain activation, the α/β allosteric antagonists stabilize the extended integrin conformation. It is interesting that the antagonists inhibit I domain activation, whereas they stabilize the rest of the integrin in the active conformation.

Most α I allosteric antagonists are highly selective for αLβ2. When α/β I-like allosteric antagonists have been tested against integrins in addition to αLβ2, cross-reactivity against αMβ2 and αXβ2 has been observed with selectivity usually close to 1 (no selectivity) and always less than 100-fold[98,100]. However, IC_{50} values differ among antagonists. It has been possible to build α-subunit selectivity into αvβ3 and αIIbβ3 antagonists as discussed above. The α/β I-like allosteric antagonists of β2 integrins seem to bind to an analogous site at the I-like domain interface with the β-propeller, and it should be possible to chemically build in selectivity for αLβ2 and αMβ2.

Like α/β I-like allosteric antagonists, antibodies to the β2 I-like domain block ligand binding indirectly[41]. However, in contrast to the α/β I-like allosteric antagonists that stabilize the I-like domain in the active conformation, inhibitory I-like domain monoclonal antibodies stabilize the I-like domain in the inactive conformation and inhibit induction of activation epitopes (A. Salas *et al.*, personal communication).

So far, all potent small-molecule antagonists of I-domain-containing integrins are allosteric inhibitors, whereas antagonists of integrins that lack I domains are ligand-mimetic, competitive inhibitors. It will be interesting to see whether potent, competitive small-molecule antagonists of I-domain-containing integrins can be discovered.

Other integrins

The integrins reviewed above are sufficient to illustrate the three different classes of integrin antagonists to date: α/β I-like competitive antagonists, α/β I-like allosteric antagonists, and α I allosteric antagonists. These integrins were chosen because they illustrate particularly well the mutually beneficial relationship between integrin drug discovery and our understanding of integrin structure and function. Nonetheless, it should be emphasized that there are other integrins that are important therapeutic targets. Among these are α4β7 and α1β1, which will be covered very briefly.

α4β7 overlaps to some extent with α4β1 in specificity, but also recognizes mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and is important in trafficking of lymphocytes to mucosal tissues[2,82]. Studies in animal models with an antibody specific to α4β7 validate it as a target in ulcerative colitis and Crohn's disease[104]. Both selective α4β7 and dual-acting α4β1 and α4β7 small-molecule antagonists have been developed. The efficacy of an antibody to α4, which inhibits both α4β1 and α4β7, in Crohn's disease and multiple sclerosis clinical trials[80,81] indicates that dual-acting α4β1 and α4β7 small-molecule antagonists[14,17] might have an attractive clinical profile.

α1β1, in addition to α2β1, α10β1 and α11β1, contains an I domain that binds to collagen[1,78] (FIG. 1). α1β1 is expressed on chronically activated, but not on resting, T lymphocytes, and an antibody against α1β1, and to a lesser extent α2β1, shows efficacy in a wide variety of animal models of autoimmune disease[105]. Apparently, α1β1 is important in T-cell migration or co-stimulation after these cells enter inflammatory sites. It should be possible to develop α/β I-like allosteric antagonists that block activation of the α1 I domain, in a manner analogous to those that block β2 integrins. Indeed, the chemical relationship described above between α4β1 α/β I-like competitive antagonists and αLβ2 α/β I-like allosteric antagonists suggests that α4β1 competitive antagonists would be good starting points for the development of α1β1 α/β I-like allosteric antagonists.

Concluding perspectives

Integrin antagonists are already well established as therapeutics for cardiovascular disease, and other applications including inflammatory disease seem extremely promising. Work with small-molecule antagonists has enhanced our understanding of the multiple structural linkages between integrin domains that enable bi-directional communication between the ligand-binding site and the cytoplasmic domains, and which regulate transitions between the bent, low-affinity conformation and the extended, high-affinity conformation. Conversely, structural studies on integrins, and mutational studies that stabilize particular conformations of integrins, have yielded important insights into the binding sites for antagonists and their mechanisms of action. The finding that both competitive antagonists and allosteric antagonists affect integrin conformation has important clinical implications. Most antagonists stabilize the extended,

high-affinity integrin conformation, in which neoepitopes are exposed. However, one class of antagonists of the integrin I domain stabilizes the bent, low-affinity conformation. It should be possible to develop a new class of antagonists of integrins lacking I domains that selectively bind to and stabilize the bent conformation; for example, compounds that would bind to and stabilize the extensive inter-domain interfaces that are present in the bent, but not in the extended, integrin conformations[23]. Further structural work on integrins and their complexes should accelerate the development of current and novel classes of integrin antagonists.

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Summary

- Members of the integrin family of adhesion molecules are non-covalently-associated α/β heterodimers that mediate cell–cell, cell–extracellular matrix and cell–pathogen interactions by binding to distinct, but often overlapping, combinations of ligands.
- Dysregulation of integrins is involved in the pathogenesis of many disease states, from autoimmunity and thrombotic vascular diseases to cancer metastasis, and so extensive efforts have been directed towards the discovery and development of integrin antagonists for clinical applications. Integrin antagonists are already well established as therapeutics for cardiovascular disease, and applications in other therapuetic areas, including inflammatory disease, seem extremely promising.
- Integrin ligand-binding function is tightly linked to molecular conformation. On activation, dramatic rearrangements occur in the overall spatial relationships of integrin domains. Understanding the structural basis of integrin activation in detail is essential for understanding the mechanism of antagonism by therapeutics, as well as for the design of second-generation antagonists with novel mechanisms of action.
- This review discusses examples of the three different classes of integrin antagonists discovered so far: α/β I-like competitive antagonists, α/β I-like allosteric antagonists and α I allosteric antagonists. These examples were chosen because they illustrate particularly well the mutually beneficial relationship between integrin drug discovery and our understanding of integrin structure and function.